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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
**C12N 15/12, A61K 45/05, C12N 15/02, G01N 33/569, C12N 5/12**

A1

(11) International Publication Number: **WO 95/16775**

(43) International Publication Date: 22 June 1995 (22.06.95)

(21) International Application Number: PCT/US94/14297

(22) International Filing Date: 14 December 1994 (14.12.94)

(30) Priority Data:

| | | |
|---|---|---|
| 08/167,464 | 14 December 1993 (14.12.93) | US |
| 08/260,547 | 16 June 1994 (16.06.94) | US |

(60) Parent Applications or Grants
(63) Related by Continuation

| | |
|---|---|
| US | 08/260,547 (CIP) |
| Filed on | 16 June 1994 (16.06.94) |
| US | 08/167,464 (CIP) |
| Filed on | 14 December 1993 (14.12.93) |

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(81) Designated States: CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

**Published**
*With international search report.*
*Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.*

(54) Title: TUMOR CELL FUSIONS AND METHODS FOR USE OF SUCH TUMOR CELL FUSIONS

(57) Abstract

The invention features products and methods for inducing an immune response against a tumor cell by providing to a patient a cell fusion product. By "cell fusion product" is meant a cell membrane from a tumor cell fused to a cell membrane from a second cell that has a greater immunogenic potential than the tumor cell.

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# TUMOR CELL FUSIONS AND METHODS FOR USE OF SUCH TUMOR CELL FUSIONS

## FIELD OF THE INVENTION

The present invention relates to products and methods useful for tumor immunotherapy.

## BACKGROUND OF THE INVENTION

A major objective in the field of tumor immunotherapy is the development of strategies for enhancing tumor immunogenicity, with potential applications for both tumor prevention and cure. To date, various products and methods useful for enhancing tumor immunogenicity have been reported. However, the methods described below are not admitted to represent prior art to the pending claims.

In general, tumors that arise de novo are poorly immunogenic, thereby escaping host antitumor responses (Hewitt et al., 33 Br. J. Cancer 241, 1976).

Methods that have been described for enhancing tumor immunogenicity include: (1) using mutagen or drug treatment (Van Pel and Boon 79 Proc. Natl. Acad. Sci. USA 4718, 1982 and Frost el al., 159 J. Exp. Med. 1491, 1984); (2) transfecting with a foreign gene encoding an exogenous antigen such as influenza hemagglutinin (Fearon et al., 38 Cancer Res. 2975, 1988); (3) reducing the expression of certain molecules in a tumor that regulate its differentiation state (Tykocinski & Ilan, 259 Science 94, 1993); (4) transferring a gene expressing a lymphokine into a tumor, for example, interleukin-2 (Fearon et al.,

60 Cell 397, 1990), interleukin-4 (Tepper et al., 57 Cell 503, 1989, Golumbek et al., 254 Science 713, 1991), interleukin-6 (Mullen et al., 52 Cancer Res. 6020, 1992), interleukin-7 (McBride et al., 52 Cancer Res. 3931, 1992); (5) transferring a gene expressing a cell surface-associated costimulator into a tumor, for example, B7 (Chen et al., 71 Cell 1093, 1992; and Townsend et al., 259 Science 368, 1993); (6) transferring a gene expressing major histocompatibility complex protein into a tumor cell; (7) transferring a gene expressing a protein that enhances the expression of a major histocompatibility complex protein in a tumor cell; and (8) transferring a gene expressing a heat shock protein into a tumor cell (Luckacs et al., 178 J. Exp. Med. 343, 1993).

Antigen-presenting cells (APC) provide molecular signals including signals mediated by APC-derived soluble cytokines and APC-derived cell surface costimulators such as: (1) B7 (Linsley et al., 87 Proc. Natl. Acad. Sci. U.S.A. 5031, 1990); (2) ICAM-I (van Seventer et al., 144 J. Immunol. 4579, 1990); (3) VCAM-I (van Seventer et al., 174 J. Exp. Med. 901, 1991); (4) LFA-3 (van Seventer et al., 21 Eur. J. Immunol. 1711, 1991); and (5) fibronectin (Shimizu et al., 145 J. Immunol., 59, 1990; Nojima et al, 172 J. Exp. Med. 1185, 1990; and Davis et al., 145 J. Immunol. 785, 1990).

A tumor cell, once appropriately modified through genetic manipulation, can itself function as an APC (Chen et al., 71 Cell 1093, 1992; Townsend et al., 259 Science 368, 1993; Tykocinski & Ilan, 259 Science 94, 1993).

## SUMMARY OF THE INVENTION

The present invention provides products and methods useful for enhancing tumor immunogenicity. More specifically, the present invention provides a cell fusion

product, and methods of using the cell fusion product to enhance tumor immunogenicity.

The general usefulness of this technology relates to the prevention and treatment of various diseases, including cancer. The disease may be present in any animal, including a human. This technology further embraces a wide range of utilities including inducing the production of antibodies in vitro. The present invention may also be used to induce the production of antibodies in a variety of animals, including humans.

It has surprisingly been discovered that the immunogenicity of a tumor cell can be remarkably enhanced by fusing a membrane of a tumor cell to a membrane from a another cell with greater immunogenic potential than the tumor cell. The phrase "cell fusion partner" is used to describe any other cell with a greater immunogenic potential than the tumor cell. Membrane extracts or whole cells may be fused in the present invention.

It is believed that fusion of a membrane from a tumor cell with a membrane from a cell fusion partner changes the capacity of the tumor cell to activate specific T-cell responders in the host immune system so that an immune response can be mounted against that tumor cell. In support of this, it has been discovered that the introduction of a hybrid cell comprising a tumor cell fused to a cell fusion partner not only reduces tumor growth, but also the growth of normal tumor cells (i.e., non-fused tumor cells) within the same host.

It is further believed that the cell fusion partner contributes relevant tumor-specific antigens to the hybrid cell and that the cell fusion partner contributes cell surface costimulators, soluble cytokines, MHC molecules, and other undefined molecular factors to the hybrid cell. These contributions in aggregate result in a highly antigenic and immunogenic phenotype for the hybrid cell. In turn, the hybrid cell can be used as an

effective cellular vaccine. However, this proposed theory is not meant to act as a limit to any alternative theories or mechanisms of carrying out the invention.

The present invention is based upon the finding that enhanced immunogenicity can be induced by fusing a cell membrane from a tumor cell with a cell membrane from a cell fusion partner, preferably one capable of effective antigen presentation and T-cell activation. Through such cell fusion, the full complement of molecular factors that are normally produced by APC's and that are required for effective T-cell activation are combined with the full set of potential tumor antigens associated with a particular tumor. Moreover, through the choice of fusion partners, it is possible to tailor the nature of the anti-tumor T-cell immune response.

Thus, a broad means has been discovered by which the antigenicity or immunogenicity of any tumor cell can be increased by fusion with a membrane from another cell with greater immunogenic potential than the tumor cell. Given this discovery and the methodology provided herein (in which examples of this discovery are provided), it is now straightforward for those in the art to screen any particular target tumor cell to determine whether fusion with any particular cell fusion partner will enhance the tumor cell's immunogenicity.

Thus, in a first aspect, the invention features methods for inducing immunity against a tumor cell by providing to a patient a cell fusion product.

By "cell fusion product" is meant a cell membrane from a tumor cell fused to a cell membrane from another cell that has a greater immunogenic potential than the tumor cell. One type of cell fusion product is a hybrid cell. By "hybrid cell" is meant a cell which is derived from the fusion of two parental cells, and it is either the direct fusion product or a daughter cell that is derived by cell division from the original fusion

product. A hybrid cell contains one or more molecular components of each of its parental cells. By "parental" cell is meant either component of the hybrid cell and includes a tumor cell or another cell with greater immunogenic potential than a tumor cell.

By "provide" is meant any method that results in the presence of a cell fusion product in a patient. The step of "providing" can be performed in a variety of ways including either administering a cell fusion product that was formed ex vivo or fusing the membranes in vivo.

By "immunity" is meant the state of being refractory to a specific disease, which is mediated by the immune system or a state of not being susceptible to the invasive or pathogenic affects of potentially infective microbes or to the affects of potentially toxic antigenic substances. By "immune response" is meant the response of the whole or part of an immune system of an organism. This response could include the activation of cellular or humoral systems, including B-cells, and T-cells.

By "tumor" is meant a collection of cells, usually dysfunctional, due to abnormal proliferation. Benign tumors are not life threatening, e.g., warts. Malignant tumors are potentially lethal cancers. All tumor types may be treated using the methods of the present invention, since cell fusion is not dependent upon any particular cell phenotype. The tumor cell may be autologous, heterologous, cultured, primary, or metastatic.

By "autologous" is meant that a tumor cell is from the patient to be treated, or from another patient having a common major histocompatibility phenotype. By "primary" is meant that a tumor cell from the organ of tumor origin in the patient to be treated is used. It also means a primary cultural cell, as distinct from a cell line. By "metastatic" is meant that the tumor cell is proliferating at sites distant from the organ of tumor

origin. By "heterologous" is meant that a tumor cell from another patient is used. Clinicians and others skilled in the art are able to identify patients in need of treatment using procedures that are well known and routine in the art. Procedures for obtaining a tumor cell from such a patient and for culturing such a cell are also well known and routine in the art. By "patient" is meant any animal, including a human, with a tumor.

By "fusing" is meant a process whereby the cell membranes are combined into a single membrane and a cell fusion product is formed. The fusing may be performed by directly injecting the cell fusion partner into a tumor mass in a patient. This may further involve identifying a patient in need of tumor therapy, obtaining a tumor cell from the patient, and culturing the tumor cell. The fusion step may involve more than two fusion partners. The fusion step may also involve the use of a chemical fusogen, such as polyethylene glycol, or electrofusion.

By "immunogenic potential" is meant the capacity to activate specific T-cell responders in the immune system or the ability to raise an immune response in an animal, preferably a human. It is commonly found that tumor cells have poor immunogenic potential relative to APCs. By "anti-tumor response" is meant any response that measurably reduces the size of a tumor, including the complete destruction of the tumor.

By "membrane extract" is meant an extract of a cell enriched for membranes, but not necessarily containing only membranes. Such an extract is chosen because it will have the antigenic and immunogenic properties necessary to induce an immune response to the tumor cell in vivo or ex vivo. A cell membrane extract may be derived from an autologous or heterologous tumor cell or another cell with greater immunogenic potential than the tumor cell. The tumor cell or cell fusion product may be treated, for example by irradiation, to

reduce its ability to proliferate. By "membrane" is meant a sheet, usually about 10 nm thick and normally composed of a bimolecular layer of lipid and protein, enclosing or partially enclosing a cell, organelle, or vacuole. Cell fusion products may be formed using less than an entire membrane, for example, portions of membranes may be used.

In other aspects the invention features methods for inducing immunity against a tumor cell by providing to a patient either a tumor cell fused to an APC or the fused membranes of such cells. Examples of a conventional APC include an activated B-cell, a dendritic cell, a macrophage, an activated T-cell, or an endothelial cell. Methods for identifying other cell fusion partners that can confer enhanced immunogenicity to a tumor cell are defined herein. Preferred candidate cells are those for which there is evidence of immunogenic potential.

In yet other aspects the invention features methods for inducing immunity against a tumor cell by providing to a patient a tumor cell fused to an activated B-cell with an artificial adhesin. By "artificial adhesin" is meant a genetically engineered molecule that is expressed through gene transfer, or through protein transfer is exogenously coated, on the surface of a cell and thereby promotes adhesion to another cell expressing a molecule on its surface that can bind to the engineered molecule. Examples of artificial adhesins include a glycosyl-phoshatidylinositol-modified polypeptide or a biotin-lipid conjugate, or other compounds with equivalent properties.

In other aspects, the invention features methods for inducing immunity against a tumor cell by providing to a patient a T-cell activated by contact with a cell fusion product. The T-cell may be part of an immunoselected subset such as CD8-positive T-cells. T-cells are known to be critical mediators of anti-tumor immunity. In general, T-cells are activated by cells collectively referred to as

"antigen-presenting cells" (APC). The diverse cell types that comprise this category share the ability to present antigens, via their major histocompatibility molecules, to the T-cell receptors on antigen-specific T-cells.

The present invention discloses the capacity of a cell fusion product to stimulate anti-tumor T-cells. This capacity can be utilized not only for in vivo stimulation of T-cells, but also for ex vivo stimulation of T-cells. Ex vivo stimulation of a T-cell using a cell fusion product can be used as a means of amplifying T-cells with tumor specificity prior to infusion of such T-cells into patients. Methods are well known in the art for delivering T-cells into patients. Methods are well known in the art for deriving T-cells from the peripheral blood of cancer patients or isolating infiltrating T-cells directly from tumors and nonspecifically amplifying their cell numbers using reagents such as interleukin-2. Contacting such T-cells with a cell fusion product offers a means for selectively amplifying the tumor-specific T-cells out of the mixed T-cell populations at these sites. Once amplified, the T-cells can be re-infused into a patient. It should be evident from this that the same patient can be coordinately treated with a cell fusion product, as an active vaccine, along with ex vivo amplified T-cells, as a passive vaccine. This combined treatment maximizes therapeutic effects and is advantageous for immunosuppressed patients.

In still other aspects, the invention features a method for identifying a cell fusion partner that can be fused to a tumor cell to generate a hybrid cell with greater immunogenicity than the tumor cell. This method involves fusing a tumor cell with a candidate cell and determining the immunogenicity of the resulting cell fusion product. Such methods can be based in vivo or ex vivo. Clinicians and others skilled in the art are able

to determine the immunogenicity of a cell using procedures that are well known and routine in the art.

In other aspects, the invention features a method for fusing a tumor cell with another cell. This method involves expressing an artificial adhesin on either the tumor cell, the other cell, or both. The method also involves combining the cells with a fusogenic agent. By "fusogenic agent" is meant any compound that increases the ability of a membrane from a tumor cell to fuse with a membrane from another cell that has greater immunogenic potential than said tumor cell.

The present invention discloses that selectivity can be conferred to a cell fusion process by inducing relevant paired cells to adhere to each other prior to addition of a fusogenic agent. By combining such a "pre-adhesion" step with subsequent fusion at low cellular densities (generally below $10^5$ cells/ml), more efficient fusion can be achieved. However, use of low cell density is not a required parameter in this invention. As described, a preferred method for achieving such pre-adhesion is through the use of an artificial adhesin that has been delivered to a relevant cell surface by any one of a number of gene and/or protein transfer methods. However, alternative methods that do not involve artificial adhesins can be used to achieve pre-adhesion. For example, a heterobifunctional antibody can be used to adhere a tumor cell and an APC. The adherence-inducing method should not perturb the antigenicity and immunogenicity of the hybrid cell to be used as a membrane source for immunization.

In still other aspects, the invention features an immunogenic cell fusion product. In this product a membrane from a tumor cell is fused to a membrane from a another cell with greater immunogenic potential than the tumor cell.

The immunogenic cell fusion products of the present invention are distinct from hybridomas used for monoclonal antibody production where a cultured myeloma cell is fused with a splenocyte. The immunogenic cell fusion product of the present invention is also distinct from non-immunogenic hybrid cells used in routine laboratory experiments. However, the methods described above for inducing immunity against a tumor cell can utilize these hybridoma and hybrid cells. In the present invention, the membrane of a tumor cell isolated from a patient is fused with a membrane from another cell, such as an APC, with greater immunogenic potential than the tumor cell. Thus, unlike monoclonal antibody production, a primary tumor cell is involved in the fusion process. Furthermore, unlike myelomas which have the ability to produce antibodies, the tumor cell of the present invention need not be able to make antibodies. At any rate, the cell fusion products of the present invention generally exclude the use of a myeloma cell fused to a splenocyte.

One or more of the cells that generate the cell fusion product, or alternatively the cell fusion product itself, may be molecularly modified prior to administration to a patient. Molecular modifications can be directed toward any one of a number of functional endpoints, including enhancing the fusion process, promoting selective adhesion between the parental cells, altering the in vivo tissue targeting properties of the cell fusion products, and further enhancing the immunogenicity of the cell fusion product above and beyond the enhanced immunogenicity that would otherwise be conferred to the hybrid cell by the parental cell.

A cell fusion partner may be modified prior to fusion with a tumor cell in order to increase the immunotherapeutic efficacy of the resulting hybrid cell. This modification can be effected by alternative methods,

including gene or protein transfer. Examples of proteins that can be expressed or inhibited in an APC include, a cell surface costimulator, a soluble cytokine, a selectin, an adhesin, a major histocompatibility complex protein, and a coinhibitor (e.g., CD8). By "foreign protein" is meant a protein that is not normally expressed in a particular cell. By "natural cell surface molecule" is meant any molecule that is naturally found on the surface of a particular cell.

One molecular modification entails the coating of one or more of the parental cells with artificial adhesins that promote selective adhesion between the two cell types prior to the fusion event. A tumor cell or another cell may be modified to enhance its fusion potential. The tumor cell can be modified in vivo or in vitro prior to cell fusion. When another cell is directly injected into a tumor mass, prior to injection the tumor mass may be modified in vivo, or the other cell may be modified in vitro, or both.

As will be readily apparent to one skilled in the art, the useful in vivo dosage to be administered and the particular mode of administration will vary depending upon the age, weight and mammalian species treated, the particular cellular compositions employed, and the specific use for which these cellular compositions are employed. The determination of effective dosage levels, that is the dosage levels necessary to achieve the desired result, will be within the ambit of one skilled in the art.

The present invention also provides kits including materials used in cell fusion.

As can be seen from the above description, the invention generally features generation of a fusion product, e.g., through cell fusion, to provide a reagent suitable for immunization against a tumor, either in a prophylactic or treatment procedure, and features methods

for identifying the optimal cell fusion partner to be fused with a tumor cell derived from such a tumor.

Prior to this invention, it is believed that no art described how tumor cell immunogenicity may be enhanced by fusion with another cell with greater immunogenic potential than the tumor cell. The use of cell fusion provides substantial advantages for practicing tumor cell engineering and enhancing tumor cell immunogenicity. Advantages of cell fusion over gene transfer include, but are not limited to, the following:

First, the present invention obviates the need for decoding the precise molecular signaling systems for individual tumor cell:T-cell combinations. Thus, unlike current methods which focus upon individual defined molecules, the present invention bypasses the general lack of understanding of the composite set of antigenic peptides and costimulatory molecules required for effective anti-tumor T-cell responses.

Second, cell fusion is applicable to diverse tumor cell types. Unlike gene transfer, cell fusion is not dependent upon the proliferative potential of the tumor cell, and hence, can be applied to a variety of tumor types which grow poorly in primary culture.

Third, cell fusion is a relatively rapid process and does not impose a burden of excessive cell culturing, shortening the interval between biopsy and treatment. Certain gene transfer-based immunotherapeutic strategies require selection for stable transfectants. This can be a time consuming process and complicates the clinical practice of such methods and imposes a delay period between biopsy and treatment.

Fourth, no biosafety hazards are known to be associated with the cell fusion method of the present invention. Gene transfer is dependent in most instances upon genetic vectors comprising viral components which carry with them some degree of biosafety hazard.

It is believed that fusing a tumor cell with another cell with greater immunogenic potential than the tumor cell effectively combines the antigenic repertoire of such a tumor cell with the multiple molecular factors, including cell surface costimulators, soluble cytokines, and major histocompatibility complex (MHC) molecules, of such other cell. Different potential other cells (including different well known APCs such as an activated B-cell, a macrophage, and an activated endothelial cell) differ in their molecular factor composition. Thus, by pairing a particular APC with a particular tumor cell, one can confer to such a tumor cell desired properties.

Cell fusion, as a means of enhancing tumor immunogenicity according to the present invention, may be combined with other known methods for enhancing tumor immunogenicity, including those cited herein, for example, expression of an exogenous gene in a tumor cell or inhibition of an endogenous gene in a tumor cell. Indeed, it is believed appropriate to combine the therapies or methods described herein with other methods to enhance the immunogenicity of a tumor cell.

The summary of the invention described in detail above is not intended in any way to limit the scope of the present invention, which is defined in the appended claims. Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

## BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows schematic diagrams of the pM-CSF-GPI/REP4$\alpha$ and pM-CSFR/REP7$\beta$ expression constructs which encode members of an artificial adhesion pair. For the pM-CSF-GPI/REP4$\alpha$ expression construct (panel a), the M-CSF-DAF chimeric sequences (thicker box) are depicted in the multiple cloning site of pREP4$\alpha$. For the pM-CSFR/REP7$\beta$ expression construct (panel b), the M-CSF

receptor coding sequence (thicker box) is depicted in the pREP7$\beta$ vector. Abbreviations: EBV oriP, Epstein-Barr virus origin of replication; EBNA-1, EBV nuclear antigen-1; RSV 3'LTR, Rous sarcoma virus 3' long terminal repeat promotor; PA, SV40 polyadenylation/termination sequences; hph, hygromycin-B resistance gene; Amp, ampicillin-resistance gene.

## DESCRIPTION OF THE PREFERRED EMBODIMENTS

Preferred embodiments of the present invention are described in detail below. However, the following description of the preferred embodiments is not intended to limit, in any way, the scope of the present invention which is defined in the appended claims.

The present invention addresses the need for conferring a complex phenotype, immunogenicity, to diverse types of tumor cells. Methods are provided for conferring this complex phenotype to tumor cells ex vivo and in vivo. In addition, methods are provided for using modified tumor cells to generate T-cells to be used for cell transfer. Yet other methods are provided for enhancing adhesion between a tumor cell and another cell, as a prelude to fusion of the two cells.

The present invention entails the fusion of a tumor cell to another cell. Methods designed to promote intercellular adhesion can be combined with conventional non-selective cell fusion methods in order to selectively target and enhance the cell fusion process.

More specifically, when tumor cells and APCs are combined, nonselective fusion agents that are routinely used to fuse cells will not only induce tumor cell:APC fusions, but also tumor cell:tumor cell and APC:APC fusions as undesired byproducts. Herein disclosed is a cell fusion method for minimizing these undesired fusion events and simultaneously maximizing the desired tumor cell:APC fusion events. This is accompanied by

artificially promoting adhesion between a tumor cell and an APC, without influencing self-adhesiveness, and adding fusing agents or performing electrofusion when adherent tumor cell:APC conjugates are at low cell densities. The lower cell densities would be less favorable to fusion between nonadherent cells of the same cell type.

According to the present invention, a combined adhesion/fusion method for generating tumor cell:APC hybrids can comprise any one of a number adhesion and fusion component methods. A method for altering the adhesive properties of cells that is particularly well-suited for the adhesion/fusion method has been developed. This method is based upon the use of a class of molecules that can be designated by the term "artificial adhesins."

Specifically, in one example, a glycosyl-phosphatidylinositol (GPI)-modified variant of the cytokine macrophage colony stimulating factor (M-CSF), designated M-CSF-GPI, was expressed on the surface of human bone marrow stromal cells. A chimeric M-CSF:decay-accelerating factor expression construct was used for M-CSF-GPI expression. Cell:cell binding assays established that this artificially membrane-tethered cytokine functioned as a potent cellular adhesin, allowing for enhanced binding to M-CSF receptor-expressing cellular transfectants. Antibody blocking analyses confirmed the M-CSF:M-CSF-receptor dependence of the enhanced intercellular binding. This capacity to direct the cellular interactive repertoire of selected cells can in principle be applied to other cell types and other molecular pairs to be used in cell-based therapies.

Intercellular adhesion is mediated by homotypic and heterotypic molecular interactions at membrane interfaces. There is a growing compendium of cell surface molecules that have been assigned functions as natural adhesins in regulatory interactions between cells. Since natural adhesins have frequently been found to be

multifunctional, they often cannot be used as neutral molecules for altering cellular adhesive properties. Moreover, most natural adhesins, by virtue of being transmembrane hydrophobic peptide-anchored molecules, can only be expressed on cell membranes by gene transfer. Therefore, methods are needed to artificially modify adhesiveness between cells in a neutral way and without necessarily using gene transfer.

A known method for accomplishing this is through the use of a palmitate-conjugated antibody as an artificial adhesin for cross-linking cells (Colsky et al., 124 J. Immunol. Methods 179, 1989). However, this approach is limited by a number of factors including the requirement for working with multichain antibody units. Soluble antibodies with bifunctional specificities offer another potential approach for cross-linking cells, but there is still the potential of signal transduction mediated by the antibodies.

There is herein disclosed an alternative approach for artificially enhancing adhesiveness between cells. Genetically engineered variants of known ligand receptor pairs can be used as artificial adhesins. The strategy is to genetically alter a soluble polypeptide ligand so that it incorporates into the surface of one cell via a carboxy-terminal anchoring domain and yet still retains its capacity to bind its receptor on another cell. The results described below document the feasibility of such an approach.

The use of a GPI moiety for membrane anchorage offers special potential advantages in the context of cellular engineering. Notably, it builds upon the demonstration (Tykocinski et al., 85 Proc. Natl. Acad. Sci. USA 3555, 1988) that any polypeptide can be readily produced as a GPI-modified variant through the use of chimeric coding sequences encompassing both the coding sequence for the protein of interest and the GPI signal

sequence from a naturally GPI-modified protein such as decay accelerating factor (DAF).

Another potential advantage of the use of GPI anchors for adhesins stems from the fact that purified GPI-modified proteins, by virtue of their amphophilic properties, can be readily reincorporated into cell membranes in the presence of low, non-lytic concentrations of non-ionic detergents such as NP-40 (Medof et al., 160 J. Exp. Med. 1558, 1984) or even in the absence of detergent. Hence, cells can be coated with purified GPI-modified polypeptides, representing a form of "protein paint," bypassing the requirement for gene transfection into the cell whose surface is being molecularly engineered. Delivery of exogenous polypeptides to cells by such a protein transfer approach circumvents problems associated with gene transfer, particular in the case of primary, nontransformed cells which in general cannot be easily transfected.

Numerous applications for this artificial adhesin technology can be envisioned, some with therapeutic implications. For example, the immunostimulatory and/or effector properties of cells used in cell-based therapies, such as tumor cell:APC hybrids, APCs, immunogenic tumor cells, or T-cells, could be selectively enhanced by increasing their adhesive properties in a selective way.

A preferred protein transfer method for coating cells with artificial adhesins involves the use of GPI-modified proteins. Methods for performing protein transfer using GPI-modified proteins have been described. In all instances, this has entailed the use of dilute concentrations of non-ionic detergent, for example, .004% NP-40, in the solution containing the GPI-modified proteins. It has been discovered that protein transfer can be accomplished even more effectively in the absence of any detergent. By leaving out the detergent, higher

concentrations of a GPI-modified protein can be used and problems associated with cell lysis by detergent are avoided. The optimal time for co-incubation of a cell with a GPI-modified protein, such as an adhesin-GPI chimeric polypeptide, is two hours at either room temperature or 37C°. 25 micrograms/ml of the GPI-modified polypeptide in the coating reaction with the cells is generally sufficient for adequate adhesin coating, although higher concentrations can be used with increased efficacy.

Another preferred protein transfer method for coating cells with artificial adhesins entails the use of a chimeric polypeptide in which an adhesin polypeptide sequence, for example, M-CSF, is linked to a streptavidin sequence. Methods for using prokaryotic expression systems to quantitatively produce such polypeptide-streptavidin chimeras are known to those familiar with the art. In using such an adhesin-streptavidin chimera, the cell of interest is pre-coated with biotin. A useful method for pre-coating cells with biotin is through the use of biotin-lipid conjugates which can be used to pre-coat cells to high biotin densities (up to $10^7$ biotin molecules/cell). Alternatively, the cells can be chemically biotinylated using standard cellular biotinylation procedures. A chimeric adhesin-streptavidin polypeptide is added to a pre-biotinylated cell, in order to generate a cell expressing the artificial adhesin at its surface. It is generally not avidin on one cell and biotin on a second cell that are being used to bring two cells together, but instead avidin and biotin are usually simply being used on the same cell surface to deliver an adhesion to that cell's surface. Then, the same process may be applied to a second cell again, potentially using an avidin-biotin complex at that cell surface. Advantages of this method include the high surface densities of artificial adhesin that can be achieved, the feasibility

of producing large quantities of the chimeric polypeptide using a prokaryotic expression system, and the biocompatibility associated with biotin-lipid conjugates in vivo.

According to the present invention, a tumor cell can be coated with one member of an artificial adhesin pair, and a conventional APC can be coated with the other member of the pair. When cell populations of each of the cell types are combined, intercellular conjugates form, pairing a tumor cell with an APC. When an electric current is applied, or a chemical fusing agent is added, to the mixed cell population, adherent cells within conjugates preferentially fuse. Preferential fusion can be further promoted by keeping the mixed cell population at a low cell density. This will minimize the formation of extraneous tumor cell:tumor cell and APC:APC hybrids with no immunotherapeutic potential.

In the examples provided below, a preferred method for fusing cells comprises the use of polyethylene glycol as a fusing agent. Other methods of fusing cells can be used, including electrofusion or use of viruses or viral components, for example, Sendai virus, that promote cell fusion.

The invention will be more fully understood with reference to the examples which follow. The following examples are intended to illustrate the invention, but not to limit its scope which is defined in the claims appended hereto. The following examples are presented to illustrate the advantages of the present invention and to assist one of ordinary skill in the art in making and using the same, but are not intended, in any way, to otherwise limit the scope of the disclosure or the protection granted by letters patent hereon.

Example 1: Hepatocarcinoma fused to an activated B-cell lose their tumorigenicity

Activated B-cells are effective APCs (Lanzavechia, 140 Nature 1985, 1985; Ron, 138 J. Immunol. 2848, 1987; and Kurt-Jones, 140 J. Immunol. 3773, 1988). Consequently, this cell type provides an excellent APC fusion partner for the present invention. BERH-2 is a chemical carcinogen-induced rat hepatocellular carcinoma cell line from the Wistar rat. BERH-2 grows rapidly and forms tumors in the liver of syngeneic animals.

In experiments described below, BERH-2 cells were fused with activated B-cells in an attempt to enhance the immunogenicity of BERH-2 cells. The data provided herein indicates that the hybrid cells, designated BERH-2-B, became more immunogenic and less tumorigenic than the tumor cell.

Example 1A: Fusion and selection of BERH-2-B cells

Activated B-cells were obtained from the spleens of rats injected 14 days earlier with bovine serum albumin in complete Freud's adjuvant. BERH-2 cells were fused with these purified activated B-cells using polyethylene glycol, using a standard B-cell hybridoma fusion protocol. The fused cells, designated BERH-2-B, were selected by panning, first with a rabbit anti-BERH-2 antiserum and second with a rabbit anti-rat B-cell antiserum.

The rabbit anti-BERH-2 and rabbit anti-rat B-cell antisera were prepared by immunizing rabbits subcutaneously with either BERH-2 hepatoma or purified B-cells from Wistar rats in complete Freud's adjuvant. Activated B-cells were purified by panning with plates coated with purified goat anti-rat Ig antibody. After repeat boosting during two months, antiserum was collected and purified by protein G-sepharose chromatography. Finally, the antiserum was repeatedly absorbed with either BERH-2 hepatoma cells or rat B-cells.

Example 1B: Expression and characterization of antigens

Expression of class I MHC, class II MHC, B7, ICAM-1 and LFA-1 on BERH-2 cells, activated B-cells and BERH-2-B hybrid cells were assessed. Cells were washed with phosphate-buffered saline (PBS) and stained with monoclonal antibody to rat MHC class I (OX-18), MHC class II (OX-6), ICAM-1 (LA 29) or LFA-1 (WT.1) as primary antibody. To stain for rat B7, we used a chimeric protein, CTLA-4-Ig. Cells were incubated with the antibodies or chimeric protein for 30 minutes on ice. A mouse anti-human CD3 monoclonal antibody (GH3, IgG2b) and a chimeric human CD44-Ig protein were used as negative controls. Cells were washed three times. FITC-goat anti-mouse Ig or FITC-labeled rabbit anti-human Ig was used as a secondary antibody and added for another 30 minutes on ice. After washing, samples were fixed and analyzed in a FACScan flow cytometer.

Parental and hybrid tumor cells were phenotyped by immunocytochemical staining and flow cytometry. The parental BERH-2 cells expressed low levels of class I MHC antigen and ICAM-1, but lacked class II MHC antigen, LFA-1 and the costimulator B7. All four hybrid BERH-2-B cell lines displayed increased class I MHC expression. In addition, BERH-2-B hybrid cell lines expressed MHC class II antigen, ICAM-1, LFA-1 and B7. These BERH-2-B cell lines have stably expressed both tumor and B-cell antigens for more than five months.

Example 1C: Comparison of tumorigenicity of parent and hybrid cells

The tumorigenicity of parental BERH-2 and hybrid BERH-2-B cells were compared, and the survival data shows enhanced animal survival for syngeneic animals injected with BERH-2-B hybrid tumor cells as compared to BERH-2 tumor cells. Two groups of female Wistar rats (ten/group) were injected intrahepatically with $2 \times 10^6$ BERH-2 cells or $2 \times 10^6$ BERH-2-B hybrid cells.

All animals injected with BERH-2 parental cells developed liver tumors and died within 60 days. In contrast, the BERH-2-B injected rats remained tumor-free for more than 180 days. While the four hybrid cell lines lost their ability to form tumors in syngeneic rats, they were able to grow and form tumors in nude mice.

In rats injected with hybrid BERH-2-B cells, there were abundant lymphocytic infiltrates at the site of injection. The tumor infiltrating lymphocytes present at the site of BERH-2-B injection at two weeks were a combination of $CD4^+$ and $CD8^+$ T-cells. As shown by immunocytochemistry, most of the infiltrating cells were T-cells. Seventy percent were $CD8^+$ T-cells, and thirty percent were $CD4^+$ T-cells. There was no inflammatory response in animals injected with parental BERH-2 tumors.

Example 2: Hepatocarcinoma:activated B-cell hybrids can be used as a cellular vaccine to prevent and cure hepatocarcinoma

The experimental data cited above established that hepatocarcinoma cells lost tumorigenicity when fused to activated B-cells. Moreover, the finding that hybrid cells elicited a prolific T-cell response was consistent with an immunologic explanation for the loss of tumorigenicity. To substantiate this conclusion, tumor prevention and cure experiments were performed.

The findings detailed below show that rats injected with BERH-2-B hybrid cells became resistant to subsequent challenge with parental BERH-2 cells. Furthermore, these experiments established that BERH-2 hepatomas were cured by injection of BERH-2-B hybrid cells. Both $CD4^+$ and $CD8^+$ T-cells were essential for the induction of protective immunity. However, only $CD8^+$ T-cells were required for the eradication of pre-existing BERH-2 tumors.

The rats immunized with BERH-2-B hybrid cells were able to prevent tumor formation by parental BERH-2

cells. Protective immunity was induced with BERH-2-B hybrid tumor cells.

Groups of female Wistar rats (8/group) were immunized with 2 x $10^6$BERH-2-B or BERH-2 cells subcutaneously. Two weeks later, both groups of the rats were challenged with 5 x $10^6$BERH-2 cells intrahepatically.

All rats pre-injected with BERH-2-B and subsequently challenged with BERH-2 remained tumor-free for more than 150 days. In contrast, all rats pre-injected with BERH-2 cells and then challenged with the same BERH-2 cells died within 60 days.

Example 2A: Tumor cure experiments

A series of tumor cure experiments were next performed to show that immunization with BERH-2-B cells could also eradicate an established hepatoma. One set of fourteen rats were injected intrahepatically with 2 x $10^6$ parental BERH-2 cells. Ten days later, eight of the injected rats were immunized with a subcutaneous injection of 5 x $10^6$ BERH-2-B hybrid cells. These rats survived for more than 120 days.

In contrast, rats injected both times with parental BERH-2 cells all died within 42 days. A second set of rats were surgically implanted with a small fragment of BERH-2 hepatoma intrahepatically. Fourteen rats were intrahepatically implanted with a small fragment (0.3 mm x 0.5 mm) of BERH-2 tumor. Two days later, eight of the animals were injected subcutaneously with 5 x $10^6$ BERH-2-B hybrid cells. The other six rats were injected subcutaneously with same number of BERH-2 cells. Ten days later, a subset of the tumor-implanted animals were injected with BERH-2-B cells, the remaining control rats were injected with parental BERH-2 cells. Whereas all 6 rats injected with BERH-2 cells died within 50 days, only 2 of 8 rats injected with BERH-2-B hybrid cells developed tumors; the latter died at 71 and 74 days after tumor

implantation, respectively. Six of the animals lived for more than 180 days after tumor implantation.

Example 2B: Determination of type of T-cell mediation

It was next determined whether the rejection of BERH-2-B cells is mediated by $CD4^+$ and/or $CD8^+$ T-cells. Rats were depleted of $CD4^+$ cells or $CD8^+$ cells by antibody treatment prior to injection of BERH-2-B cells. BERH-2-B cells were able to form tumors in both CD4-depleted and CD8-depleted rats. The effects of depletion of CD4+ or CD8+ cells on the growth of BERH-2-B and BERH-2 tumor cells in vivo are discussed below.

Example 2C: Groups A-D

Female Wistar rats (Groups A, B, C, D) were treated with purified anti-rat CD4 (OX38), or anti-CD8 (OX-8) or a control mouse anti-diethylthiamine pentaacetic acid monoclonal antibody. Each animal received 500 $\mu$g of the purified antibody intravenously twice per week for three weeks.

Two days before injection of tumor cells, peripheral blood lymphocytes were obtained from individual treated rats and stained with monoclonal antibodies to CD4 or CD8 to verify the depletion of $CD4^+$ or $CD8^+$ cells, respectively. Treatment with anti-CD4 monoclonal antibody depleted more than 95% of the $CD4^+$ cells, and treatment with anti-CD8 monoclonal antibody depleted close to 95% of the $CD8^+$ cells; treatment with control antibody did not alter the number of $CD4^+$ and $CD8^+$ cells. Three days after the last injection of the antibodies, all rats were injected intrahepatically with $5 \times 10^6$ BERH-2-B tumor cells.

Example 2D: Groups E-G

Rats were first immunized with BERH-2-B cells and then depleted of CD4+ or CD8+ cells 14 days later. Female Wistar rats (Groups E, F, G) were first immunized with $2 \times 10^5$ BERH-2-B cells subcutaneously. Two weeks after immunization, animals were treated with anti-CD4, or

anti-CD8 or control antibody, and the effectiveness of the depletions was verified by immunofluorescence and flow cytometry. Three days after the last injection of monoclonal antibody, all animals received 5 x $10^6$ BERH-2 cells intrahepatically. These experiments have been repeated twice with comparable results.

**Table 1**

Effects of Depletion of $CD4^+$ or $CD8^+$ Cells
on the Growth of BERH-2-B and BERH-2 Cells In Vivo

| Antibody Specificity | Treatment Protocol | | Number of Animals With Tumors |
|---|---|---|---|
| | Ab treat first then immunize | immunize first then treat with Ab | |
| None | - | | 0/6 |
| CD4 | + | | 4/6 |
| CD8 | + | | 5/6 |
| Control Ab | + | | 0/6 |
| CD4 | | + | 0/5 |
| CD8 | | + | 5/5 |
| Control Ab | | + | 0/5 |

These CD4-or CD8-depleted rats were then challenged with BERH-2 cells. Tumors developed in CD8-depleted, but not in CD4-depleted rats. This indicates that whereas both $CD4^+$ and $CD8^+$ cells are necessary for the induction of protective anti-tumor immunity, once the immune response has been induced, $CD8^+$ cells are sufficient alone to mediate tumor cell destruction alone. These results contrast with those reported previously for murine melanoma cells transfected with the B7 costimulator gene where $CD4^+$ cells were not required for induction of the anti-tumor immune response (Chen, 71 Cell 1093, 1992; Townsend, 259 Science 368, 1993).

Example 2E: Tumor-specificity of immunity

It was next determined whether the immunity induced by BERH-2-B cells is tumor-specific. NBT-II is a bladder carcinoma that grows rapidly in syngeneic Wistar rats. Immunization with BERH-2-B hybrid cells prevented the growth of the parental BERH-2 cells. However, immunization with BERH-2-B was unable to inhibit the growth of NBT-II cells.

The specificity of the anti-BERH-2 immune response elicited by BERH-2-B hybrid tumor cells was documented. Female Wistar rats were injected with 2 x $10^6$ BERH-2-B cells subcutaneously. Two weeks after immunization, one group of the rats were injected with 5 x $10^6$ BERH-2 cells intrahepatically. Another group of rats were injected with 5 x $10^6$ NBT-2 rat bladder carcinoma cells (obtained from the American Tissue Type Collection).

Tumor developed locally in the injected site in rats immunized with BERH-2-B tumor cells and challenged with NBT-2 tumor cells in all eight animals. All animals in this group died within 45 days after tumor challenge. In addition, $CD8^+$ T-cells from BERH-2-B-immunized rats killed BERH-2 cells but did not kill NBT-II cells in vitro.

**Table 2**

Specificity of the Immune Response
Elicited by BERH-2-B Hybrid Tumor Cells

| Immunization | Challenge | Number of Animals With Tumors |
|---|---|---|
| BERH-2-B | BERH-2 | 0/8 |
| BERH-2-B | NBT-II | 8/8 |

Example 2F: Necessity of in vitro selection

It was determined whether the in vitro selection step for hybrid cells is required for effective induction of anti-tumor immunity.

Tumor protective immunity induced with BERH-2 tumor cells fused with activated B cells does not require in vitro selection. Three groups of rats (8/group) were injected subcutaneously with 5 x $10^6$ BERH-2 cells, 5 x $10^6$ BERH-2 cells mixed with 5 x $10^6$ activated B cells, or 5 x $10^6$ BERH-2 cells fused with 5 x $10^6$ activated B cells in the presence of PEG. Fused cells were washed three times with PBS, resuspended in PBS and injected subcutaneously. Two weeks later, all groups of rats were challenged with 5 x $10^6$ BERH-2 tumor cells intrahepatically.

BERH-2 tumor cells were fused with activated B-cells. After fusion, cells were washed and injected into syngeneic rats with in vitro selection. The efficiency of the fusion in such experiments ranged form 30% to 50%. As controls, BERH-2 tumor cells mixed with activated B-cells without PEG were injected subcutaneously. All animals were then challenged with the parental BERH-2 cells intrahepatically.

The data indicated that fused cells were immunogenic in the absence of in vitro selection. Only animals immunized with tumor cells fused with activated B-cells were able to reject the parental tumor cells; simply mixing tumor cells with activated B-cells was not effective. This finding that protective immunity can be induced by tumor cells fused with activated B-cells without an in vitro selection step simplifies the clinical therapeutic application of this method.

These findings indicate that an effective BERH-2 hepatocarcinoma-specific vaccine can be generated by fusing tumor cells with syngeneic, activated B-cells. It is believed that in addition to class II MHC and B7 costimulator, hybrid BERH-2-B cells may express other molecules that are essential for the activation of anti-tumor T-cells. This may include, but is not limited to, soluble cytokines. Production of cytokines by hybrid

tumor cells may be important in the elicitation of host immune responses.

Example 3: Glycosylphosphatidylinositol ("GPI")-modified cytokine can function as an artificial adhesin

For purposes of achieving high level stable expression of cell surface molecules on human cellular transfectants, self-replicating EBV episomal expression vectors were employed (Groger et al, 81 Gene 285, 1989). In the present study, two EBV vector variants designated pREP4$\alpha$ and pREP7$\beta$ were used, both of which share a transcriptional cassette in which the RSV 3' LTR promoter, a multiple cloning site, and the SV40 late polyadenylation/termination signal are linked in tandem.

An expression construct for M-CSF-GPI was generated as follows. The 1.8 kb M-CSF coding region fragment (XhoI - EcoRI) of p3ACSFR1 (Fig. 1) was inserted into the corresponding sites of pBluescript (pBT, Stratagene, Inc.). This generated a GPI-anchored variant of M-CSF, pM-CSF/BT was cut with NcoI, filled-in with the pollk, and subsequently cut with BamHI. The 3' AvaII (filled-in) to BamHI from the DAF subclone pDF2.1/BT was subcloned into this vector to generate an in-frame M-CSF-DAF chimeric sequence. The KpnI-BamHI fragment of the resultant plasmid containing the chimeric sequence was subcloned into the corresponding sites of pREP4$\alpha$ to generate pM-CSF-GPI/REP4$\alpha$.

An M-CSF receptor EBV episomal expression construct was generated as follows: A 4.0kb EcoRI fragment of pc-fms 102 was subcloned into the EcoRI site of pBluescript (Stratagene, Inc.) to generate pM-CSFR/BT. The 3.6 kb BamHI fragment of this subclone, containing the entire M-CSFR coding region, was subcloned in a sense orientation into the BamHI site of pREP7$\beta$ to generate pM-CSFR/REP7$\beta$.

The overall experimental strategy of this study was to use stable gene transfer to modify the adhesive

properties of cells. The paired cellular targets chosen were the human SV40 large T-immortalized bone marrow stromal cell line KM-102 and the human myeloid leukemia cell line K562. Previous work with both the KM-102 and K562 lines have shown them to be efficient transfection targets with EBV expression vectors.

With the goal of enhancing adhesion between KM-102 and K562 cells, we stably transfected them with episome-based expression constructs for an artificial GPI-modified variant of M-CSF, designated M-CSF-GPI, in which natural M-CSF coding sequence is linked in-frame to the GPI signal sequence of human DAF, into KM-102 stromal cells. Indirect immunofluorescence staining demonstrated a high level surface expression of M-CSF epitopes on the pM-CSF GPI/REP4$\alpha$ hyg$^R$KM-102 transfectants. No M-CSF epitope was detectable on the surface of nontransfected KM-102 cells or on KM-102 cells transfected with the irrelevant EBV episome pRSVCAT$\alpha$/220.2.

pM-CSFR/REP7$\beta$ and pM-CSF GPI/REP4$\alpha$ were introduced into K562 and U937 cells, respectively, by lipofection. The KM-102 stromal cell line was kindly provided by K. Harigaya and maintained in McCoy's 5a medium (Gibco, Inc.) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (M.A. Bioproducts)/10 mM HEPES/40 $\mu$g/ml gentamycin sulfate in a humid 5% $CO_2$ atmosphere at 37°C. pM-CSF-GPI/REP4$\alpha$ was introduced into KM-102 cells by lipofection. Briefly, cells were grown to 50% confluence in six-well plates, and washed twice with PBS and once with Opti-MEM (Gibco). Cells were then incubated for 5-8 hours at 37°C with 1 ml Opti-MEM, containing 10 $\mu$g DNA and 30 $\mu$g lipofectin, before adding 1 ml of complete medium containing 20% FBS.

Seventy-two hours post-transfection, selection for stable transfectants was begun by replacing the medium with fresh medium containing 75 $\mu$g/ml hygromycin B (Calbiochem, Inc.) Stably hyg$^R$ transfected colonies were

picked at 2-3 weeks using cloning rings (Bellco, Inc.) expanded and maintained in 100 μg/ml hygromycin B. Surface M-CSF expression was established by indirect immunofluorescence using a polyclonal rabbit anti-M-CSF antibody (Genzyme) and a FITC-anti-rabbit IgG (BMB) secondary antibody. M-CSFR expression was confirmed by indirect immunofluorescence and flow cytometry (FACS), using a rat monoclonal anti-M-CSFR primary antibody (Oncogene Sciences) and FITC-conjugated rabbit anti-rat IgG secondary antibody (Miles ICN).

In parallel, K562 leukemic cells were transfected with human M-CSF receptor (M-CSFR or c-fms) episomal expression construct, designated pM-CSFR/REP7β. Abundant surface expression of natural human M-CSFR was demonstrated by indirect immunostaining and flow cytometry. To control for episomal transfectants demonstrating non-specific up-regulation of their endogenous adhesion molecules, K562 cells were transfected with the irrelevant episome pRSVCATα/220.2.

To confirm that pM-CSF-GPI/REP4α yields a GPI membrane anchored product, phosphatidylinositol-specific phospholipase C (PIPLC), an enzyme which specially cleaved GPI moieties made by certain cells from their surfaces, was used. PIPLC treatment of KM-102 cells transfected with pM-CSF-GPI/REP4α did not result in a significant release of either M-CSF or DAF, another GPI-anchored protein serving as control, from the surface, as determined indirect immunofluorescence. This suggested that KM-102 is similar to some other cell types that are known to express a GPI anchor variant that is resistant to PIPLC cleavage.

PIPLC cleavage was performed by incubating 1 x $10^6$ cells with 1 unit of PIPLC (Boehringer Mannheim Biochemicals) at 37°C for one hour in RPMI 1640 medium containing 10%FBS and 0.01% sodium azide. Cleavage was assessed using an anti-DAF antibody and flow cytometry.

In light of this finding, pM-CSF-GPI/REP4α was additionally transfected into the U937 cell line which is known to produce PIPLC-sensitive GPI anchors. PIPLC susceptibility of M-CSF and endogenous DAF on these cells was assessed. Nontransfected U937 cells did not express M-CSF on their surface. The pM-CSF-GPI/REP4α U937 transfectants expressed high levels of cell surface GM-CSF. The tethered M-CSF could be specifically cleaved with PIPLC to an extent similar to that seen with endogenous GPI-anchored DAF protein. The PIPLC cleavage yielded removal of surface M-CSF epitope to the same extent as DAF. This indicates that the pM-CSF-GPI/REP4α construct generates a GPI-anchored form of M-CSF.

It was next determined whether the pM-CSF-GPI/REP4α KM-102 transfectants would bind preferentially M-CSFR$^+$ cellular targets. Cell:cell binding between adherent KM-102 cells and M-CSFR$^+$ nonadherent K562 targets cells was enhanced approximately three-fold when M-CSF-GPI was present on the surface of the KM-102 cells.

Intercellular adhesion between adherent KM-102 transfectants and nonadherent K562 transfectants was measured using a cell:cell binding assay. Normal KM-102 cells (nontransfected), a stable transfectant expressing GPI-anchored M-CSF (pM-CSF-GPI/REP4α), a control transfectant expressing the lymphoid cell surface molecule (pCD8/REP2.1), or an irrelevant transfectant (pαIL-6/REP5.1) were separately seeded into wells of a polyvinyl, flat-bottom 96-well plate. $^{35}$S-labeled K562 target cells, either nontransfected (none) or expressing the M-CSF receptor (pM-CSFR/REP7β) or CAT (pRSVCAT/220.2), were added to the wells and allowed to bind to the KM-102 cells. The number of K562 target cells which remained adherent following an inverted centrifugation was calculated by measuring the specific activity of the target cells added. A significant increase in adherence between pM-CSFR-REP4α transfected KM-102 and pM-CSFR-REPβ7

transfected KM-102 was noted whereas no increase in adhesion was noted for other combinations.

This was specific for M-CSF expression, since control KM-102 transfectants containing either pCD8/REP2.1 encoding the irrelevant surface protein CD8 or alternatively pαIL-6/REP5.1 driving antisense IL-6 RNA expression, demonstrated no enhanced binding to M-CSFR$^+$ targets. Moreover, no significant augmentation of adhesion was evident when nontransfected K562 cells, or K562 cells transfected with pRSVCATα/220.2 were used as cellular targets. However, K562 transfectants overall do appear to be slightly more adhesive than nontransfected cells.

To definitively establish that it is the membrane-associated M-CSF, and not secondary expressed surface molecules on the pM-CSF-GPI/REP4α KM102 transfectants, that was specifically responsible for the enhanced adhesion, antibody blocking analysis was performed. Prior incubation of M-CSFR$^+$ K562 target cells with antibodies directed against the M-CSFR or alternatively, of surface M-CSF$^+$ KM102 cells with polyclonal anti-M-CSF antibodies, each partially inhibited this specific cellular interaction.

The effects of blocking antibodies upon the binding of M-CSF-GPI and M-CSFR-positive cells to each other was assessed in cell:cell binding assays. Nontransfected K562 cells (none) or K562 cells stably expressing the M-CSFR (pM-CSFR/REP7β) or CAT (pRSVCAT/220.2) were allowed to bind to KM-102 cells expressing GPI-anchored M-CSF (pM-CSF-GPI/REP4α). Blocking antibodies were added to the appropriate cells, as indicated, 30 min. prior to adding the cells to the wells. Anti-M-CSF, polyclonal anti-M-CSF antibody; anti-M-CSFR, monoclonal anti-M-CSF receptor (c-fms) antibody; an anti-TRF, monoclonal anti-transferrin receptor

antibody. Normal rabbit serum (NRS) was added, as indicated, to prevent F receptor cross-linking.

The simultaneous addition of these two blocking antibodies, directed against both members of the ligand:receptor pair, completely blocked the specific binding. Normal rabbit serum, which did not inhibit binding, was included in all experiments to prevent the cross-linking of cells through Fc receptors expressed on the K562 cells. The antibody-mediated inhibition observed was specific for the M-CSF:M-CSFR pair, since antibodies against the human transferrin receptor (TFR), known to be expressed on K562 cells, had no blocking effect.

Cell:cell binding assays employed a modification of a published method (McClay et al., 78 Proc. Natl. Acad. Sci USA 4975, 1981). Briefly, 3 x $10^4$ nontransfected or transfected KM-102 cells were placed in wells of a polyvinyl, flat-bottom 96-well plate (Dynatech Laboratories) with 0.1 ml complete medium per well, and the cells were incubated at 37°C for two days. Wells were pretreated with fetal bovine serum for two hours to promote attachment of the KM-102 cells. The KM-102 cells were generally 60-80% confluent at the time of the cell:cell binding assay. The K562 target cells, labeled with $^{35}$S-methionine, were washed, resuspended in complete medium at 5 x $10^5$ cells per ml, and 0.1 ml was added directly to the wells.

The plates were incubated at 37°C for 2.5 hours to allow for maximal binding. Medium was added to each well to produce a positive meniscus, and then plates were carefully sealed with adhesive plate sealers (Dynatech Labs, Inc.). The plates were inverted and centrifuged for 10 minutes at room temperature using Sorvall micro-plate carriers. A relative centrifugal force (RCF) of 900 x g was used for most experiments. Post-centrifugation, still inverted plates were flash-frozen at -80°C, and the bottoms of each well, containing the stromal cells and

bound targets, were cut off and placed in scintillation vials for counting. The number of K562 cells bound per well was calculated as follows:

$$\text{CELLS BOUND/WELL} = \frac{\text{CPM BOUND TO STROMAL CELLS}}{\text{TOTAL CPM ADDED/WELL}} \times (5 \times 10^4)$$

Each group represents the means of at least triplicate samples. Representative KM-102 stromal cell wells were harvested and counted to ensure that equivalent cell numbers were present in each well.

For antibody blocking experiments, antibodies were added directly to cell suspensions or to wells (as indicated) and incubated at room temperature for 30 minutes just prior to the addition of target cells to the wells. The final concentrations of antibodies used in the blocking studies were: rabbit anti-human M-CSF polyclonal antibody (Genzyme), 10$\mu$g/ml; rat anti-c-fms/CSF-1 receptor (Oncogene Science, Inc.), 2 $\mu$g/ml; and mouse monoclonal anti-human transferrin receptor (Hybritech, Inc.) 8 $\mu$g/ml. Heat-inactivated normal rabbit serum (4 $\mu$l/1 x $10^5$ cells) was added in each case to prevent the cross-linking of cells through Fc receptors.

Hence, in this example, a method for selectively altering intercellular adhesion has been demonstrated. Specifically, it has shown that an artificial GPI-modified variant of a model cytokine M-CSF, when anchored at the cell surface, can augment cellular binding to M-CSF receptor-bearing tumor cells. High level expression of both the M-CSF-GPI and M-CSFR molecules on their respective cells could be efficiently obtained via gene transfer using episomal expression vectors. The M-CSF-dependence of the effect was verified by anti-M-CSF and anti-M-CSF receptor antibody blocking. Clearly, the invention is not limited to these variants since GPI-anchored variants of multiple ligands other than M-CSF could be used as alternative artificial adhesins.

In the above experiment, only the M-CSF component of the M-CSF:M-CSFR pair was GPI-anchored. The M-CSFR component could also be GPI-anchored. Together, this would permit coating one cell with a ligand:GPI (e.g., M-CSF:GPI) chimera and a second cell with a receptor:GPI (e.g., M-CSFR:GPI) chimera and thereby selectively enhancing adhesiveness between the respective cells via protein transfer. Such a use of a "disabled" GPI-modified receptor, as part of an artificial adhesin pair, would obviate the possibilities for unwanted signaling through the receptor. Although there is evidence for cis signaling through certain natural GPI-anchored proteins, it seems likely that most cytokine receptors, when artificially GPI anchored, will not be functionally responsive to their corresponding cytokines due to distortions in molecular topology.

The pre-adhesion step entails the mixing of three components, namely, a tumor cell, a second cell with greater immunogenic potential, and artificial adhesin molecules. After a co-incubation period, generally lasting greater than 20 minutes, the mixture is centrifuged at 400g for 5-10 minutes in appropriate tissue culture medium supplemented with antibiotic and resuspended in the same medium. The cell suspension is kept at room temperature for 30 minutes with gentle stirring. Subsequently the mixture is spun through a sterilized isotonic sucrose (320 mOsm) solution containing 2 mM sodium phosphate buffer, pH 7.2 (400g for 5-10 minutes). The cell pellet comprising tumor cell:second cell conjugates is gently suspended in 1 ml of isotonic sucrose buffer.

Following pre-adhesion using artifical adhesins and subsequent enrichment for heterologous cell conjugates, fusion is carried out. A preferred method for fusing the heterologous cells in conjugates is electrofusion. Methods for performing electrofusion are

well-known to those familiar with the art. Of note, most electrofusion protocols comprise two-steps: the induction of membrane-membrane contact followed by application of the fusogenic pulse. The dielectrophoresis method is used in most experiments to achieve the first step of congregating cells before fusion-inducing electric pulses are applied. However, it is known that the two steps can be dissociated, that is, one need not congregate cells by dielectrophoresis in order to achieve fusion (Sowers, 220 Methods in Enzymology 196, 1993). A preferred method according to the present invention entails omitting the dielectrophoresis step for congregating cells non-selectively, and instead applying the fusogenic pulse to heterologous cell conjugates that have been pre-adhered with artificial adhesins. The fusogenic pulse can be applied with a high voltage generator, and alternative instruments are commercially available. Though it is preferable to use conjugates at low conjugate densities (less than $10^5$ per ml), one can readily go up to $10^7$ per ml or higher in practicing this invention. Typical electrofusion conditions use five square wave pulses of 2.5 kV/cm and 5 microsecond duration, at a controlled temperature of 35C and with intervals of 15 sec between pulses to permit dissipation of Joule heat. Optimization of fusion conditions for particular cell types can be readily performed. (Less optimal adhesins are those described by Tsong and Tomita, 220 Methods in Enzymology 238, 1993).

While the present invention has been described in conjunction with the preferred embodiments and examples, one of ordinary skill, after reading the foregoing specification, will be able to effect various changes, substitutions or equivalents and other alterations to the invention provided herein. It is therefore intended that the protection granted by letters patent hereon be limited only by the definitions contained

in the appended claims, and equivalents thereof. It will be understood that changes may be made and the details of formulation without departing from the spirit of the invention as defined in the following claims.

What is claimed is:

CLAIMS

1. A method for inducing an immune response against a first tumor cell comprising the step of providing to a patient a cell fusion product, wherein said cell fusion product comprises a membrane from a second tumor cell fused to a membrane from another cell with greater immunogenic potential than said second tumor cell.

2. The method of claim 1, wherein said cell fusion product comprises a hybrid cell, wherein said hybrid cell comprises a second tumor cell fused to another cell with greater immunogenic potential than said tumor cell.

3. The method of claim 1, wherein said step of providing comprises:

forming said cell fusion product ex vivo; and

administering said cell fusion product to said patient.

4. The method of claim 1, wherein said step of providing comprises forming said cell fusion product in vivo.

5. The method of claim 1, wherein said step of providing comprises directly injecting into a tumor in said patient another cell with greater immunogenic potential than said second tumor cell.

6. The method of claim 1, further comprising the step of obtaining said second tumor cell from said patient.

7. The method of claim 6, further comprising the step of culturing said tumor cell.

8. The method of claim 7, further comprising the step of fusing said membrane from said second tumor cell with said membrane from said cell with greater immunogenic potential than said second tumor cell.

9. The method of claim 1, wherein said second tumor cell is autologous.

10. The method of claim 1, wherein said second tumor cell is a primary tumor cell.

11. The method of claim 1, wherein said second tumor cell is a metastatic tumor cell.

12. The method of claim 1, wherein said cell with greater immunogenic potential than said second tumor cell is an antigen-presenting cell.

13. The method of claim 12, wherein said antigen-presenting cell is selected from the group consisting of an activated B-cell, a dendritic cell, a macrophage, an activated T-cell, and an endothelial cell.

14. The method of claim 12, wherein said antigen-presenting cell is modified to alter its immunogenicity.

15. The method of claim 14, wherein said modification comprises expression of a foreign protein in said antigen-presenting cell.

16. The method of claim 14, wherein said modification is accomplished by gene transfer.

17. The method of claim 14, wherein said modification is accomplished by protein transfer.

18. The method of claim 14, wherein said modification comprises inhibition of a protein in said antigen-presenting cell.

19. The method of claim 18, wherein said protein is selected from the group consisting of a major histocompatibility complex protein and a coinhibitor.

20. The method of claim 15, wherein said foreign protein is selected from the group consisting of a cell surface costimulator, a soluble cytokine, a selectin, an adhesin, and a major histocompatibility complex protein.

21. The method of claim 1, wherein said second tumor cell or said cell with greater immunogenic potential than said second tumor cell is modified to enhance its fusion potential by expressing an adhesin on either of said cells.

22. The method of claim 21, wherein said adhesin is expressed on both of said cells.

23. The method of claim 1 or 22, wherein said adhesin is an artificial adhesin.

24. The method of claim 23, wherein said artificial adhesin is a glycosyl-phosphatidylinositol-modified polypeptide.

25. The method of claim 23, wherein said artificial adhesin comprises a biotin-lipid conjugate and a chimeric streptavidin polypeptide.

26. The method of claim 1, wherein said cell fusion product is made using a chemical fusogen.

27. The method of claim 26, wherein said chemical fusogen is polyethylene glycol.

28. The method of claim 1, wherein said cell fusion product is made using electrofusion.

29. The method of claim 1, wherein said second tumor cell is a heterologous tumor cell.

30. The method of claim 29, wherein said heterologous tumor cell is obtained from a cultured cell line.

31. The method of claim 29, wherein said heterologous tumor cell is a primary tumor cell.

32. The method of claim 29, wherein said heterologous tumor cell is a metastatic tumor cell.

33. The method of claim 1, wherein said membrane from said second tumor cell is derived from an autologous tumor cell.

34. The method of claim 1, wherein said membrane from said second tumor cell is derived from a heterologous tumor cell.

35. The method of claim 1, wherein said second tumor cell is treated prior to fusion to reduce its ability to proliferate.

36. The method of claim 3, wherein said cell fusion product is treated to reduce its ability to proliferate prior to administration to said patient.

37. The method of claim 35 or 36, wherein said treatment is irradiation.

38. The method of claim 5, wherein said first tumor cell of said tumor is modified in vivo prior to injection of another cell with greater immunogenic potential than said second tumor cell.

39. The method of claim 38, wherein said modification comprises expressing on said first tumor cell a molecule that promotes adhesion to said cell with greater immunogenic potential than said second tumor cell.

40. The method of claim 5, wherein said cell with greater immunogenic potential than said second tumor cell is modified in vitro prior to injection.

41. The method of claim 40, wherein said modification comprises expressing on said cell with greater immunogenic potential than said second tumor cell a molecule that promotes adhesion to said first tumor cell.

42. The method of claim 5, wherein said first tumor cell or said cell with greater immunogenic potential than said second tumor cell is modified by gene transfer.

43. The method of claim 5, wherein said first tumor cell or said cell with greater immunogenic potential than said second tumor cell is modified by protein transfer.

44. The method of claim 1, wherein said cell fusion product comprises more than two cells.

45. A method for inducing an immune response against a first tumor cell, comprising the step of providing to a patient a T-cell activated by contact with a cell fusion product, wherein said cell fusion product comprises a membrane from a second tumor cell fused to a membrane from another cell with greater immunogenic potential than said second tumor cell.

46. The method of claim 45, further comprising the step of immunoselecting a T-cell subset to provide to a patient.

47. The method of claim 46, wherein said T-cell subset consists of CD8-positive T-cells.

48. A method for inducing an immune response against a first tumor cell comprising the step of providing to a patient a second tumor cell fused by an artificial adhesin to an activated B-cell.

49. A method for identifying a cell that fuses to a tumor cell and thereby provides a cell fusion product with immunogenicity greater than said tumor cell, comprising the steps of:

fusing said tumor cell with a candidate cell; and

determining the immunogenicity of the resulting cell fusion product.

50. The method of claim 49, wherein determining the step of immunogenicity of said cell fusion product is performed by administering said cell fusion product to an experimental animal.

51. A method for identifying a cell that fuses to a tumor cell and thereby provides a cell fusion product

that induces an anti-tumor immune response comprising the steps of:

fusing said tumor cell with a candidate cell; and

determining the anti-tumor immune response of the resulting cell fusion product.

52. A method for fusing a tumor cell with another cell with greater immunogenic potential than said tumor cell, comprising the steps of:

expressing on either one of said tumor cell or said other cell an artificial adhesin that increases adhesion between said tumor cell and said other cell; and

fusing said cells.

53. The method of claim 52, wherein said artificial adhesin on one of said cells contacts a natural cell surface molecule on the other of said cells.

54. The method of claim 52, wherein said artificial adhesin on one of said cells contacts an artificial adhesin on the other of said cells.

55. The method of claim 52, wherein expression of said artificial adhesin is accomplished by protein transfer.

56. The method of claim 52, wherein expression of said artificial adhesin is accomplished by gene transfer.

57. The method of claim 52, wherein said step of fusing said cells is performed at cell densities below $10^8$ cells per milliliter.

58. An immunogenic cell fusion product comprising a membrane from a tumor cell fused to a membrane from a second cell with greater immunogenic potential than said tumor cell, but wherein said cell fusion product does not comprise a hybridoma cell.

59. The immunogenic cell fusion product of claim 58, wherein said second cell is an antigen-presenting cell.

60. The immunogenic cell fusion product of claim 59, wherein said antigen-presenting cell is selected from the group consisting of a B-cell, a dendritic cell, a macrophage, an activated T-cell, and an endothelial cell.

61. The immunogenic cell fusion product of claim 58, wherein said fusion is achieved using a heterobifunctional antibody.

FIG. 1a.

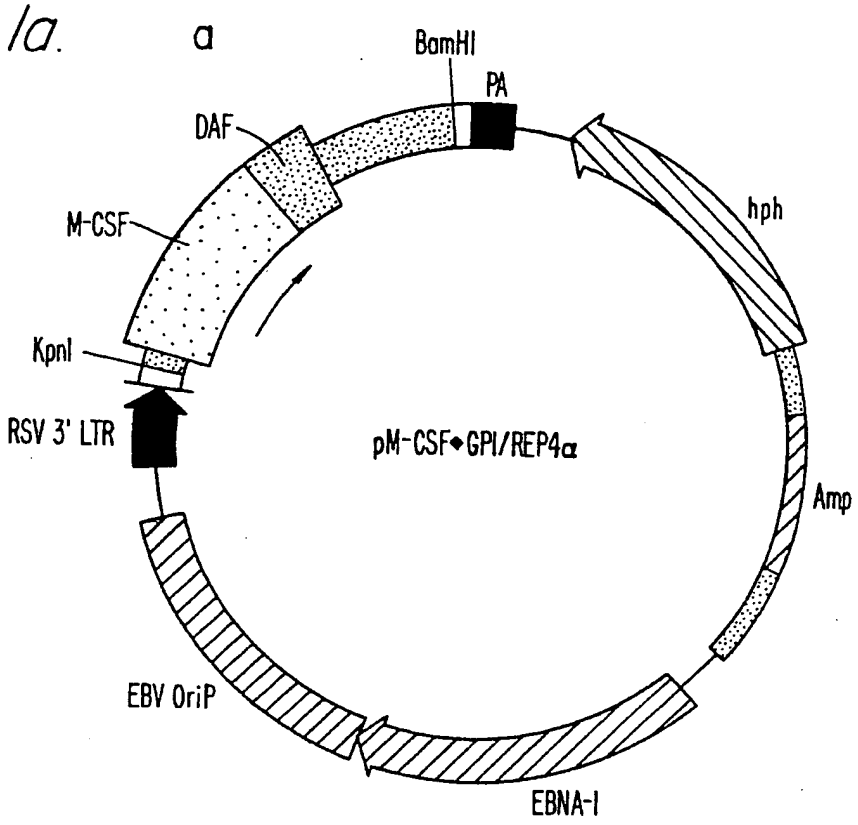
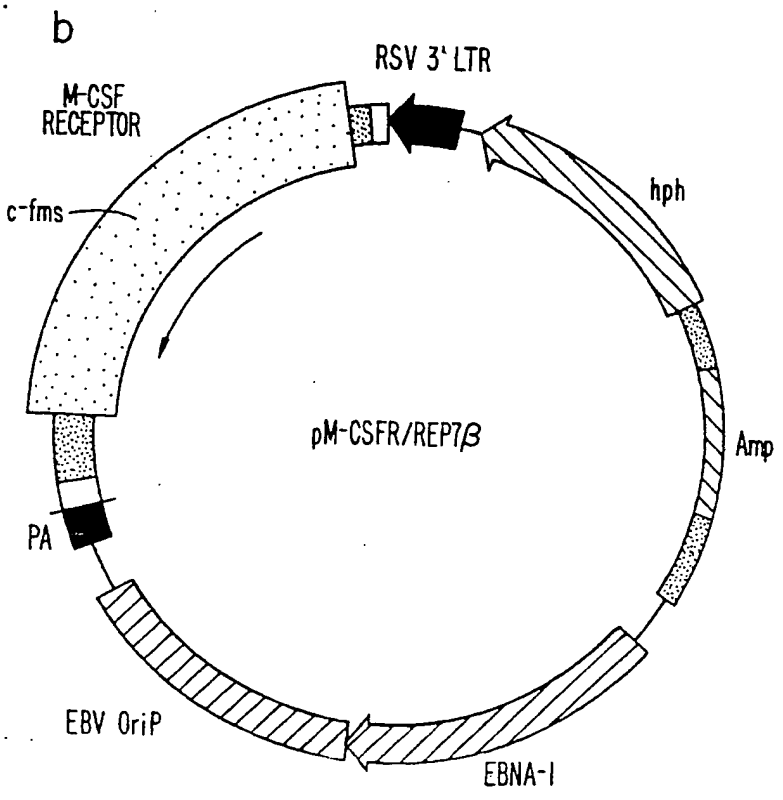

a
BamHI
PA
DAF
M-CSF
KpnI
RSV 3' LTR
hph
pM-CSF◆GPI/REP4α
Amp
EBV OriP
EBNA-I

FIG. 1b.


b
RSV 3' LTR
M-CSF
RECEPTOR
c-fms
hph
pM-CSFR/REP7β
Amp
PA
EBV OriP
EBNA-I

# INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/US 94/14297

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 A61K45/05 C12N15/02 G01N33/569 C12N5/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | IN VIVO, vol. 4,no. 3, May 1990 ATHENS, GREECE, pages 185-190, A. GOGUEL ET AL. 'MHC class I antigen expression and alterations induced by rIFNgamma in tumor hybrid cell immunogenicity.' see the whole document --- -/-- | 1-3,6-8, 12,14, 26,27, 29,30, 34, 49-51, 58-60 |

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 19 April 1995 | 04. 05. 95 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax (+ 31-70) 340-3016 | Nooij, F |

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | INTERNATIONAL JOURNAL OF CANCER, vol. 32,no. 4, 15 October 1983 GENEVA, SWITZERLAND, pages 507-514, K. KAWASHIMA ET AL. 'High-grade tumor-specific immunity induced by L1210 leukemia variants obtained from the culture of L1210 cells fused with Lesch-Nyhan fibroblasts.' see the whole document | 1-3,6-8, 12,26, 27,29, 30, 34-37, 49-51, 58-60 |
| X | IMMUNOLOGY, vol. 52,no. 2, June 1984 OXFORD, GB, pages 281-290, R. SLOMSKI ET AL. 'Surface antigens of immunoprotective leukaemia x fibroblast hybrid cells which have lost malignant properties in histocompatible mice differ from the malignant parental cells.' see the whole document | 1-3,6-8, 12,26, 27,29, 30, 34-36, 49-51, 58-60 |
| X | EP,A,0 203 403 (ASAHI KASEI KOGYO KK) 3 December 1986 see example 4 see claims 3-11 | 58-60 |
| Y | | 1-3, 6-16,20, 26,27, 29-34 |
| Y | WO,A,93 07906 (SAN DIEGO REGIONAL CANCER CENTER) 29 April 1993 see examples see claims | 1-3, 6-16,20, 26,27, 29-34 |
| A | SCIENCE, vol. 259,no. 5093, 15 January 1993 WASHINGTON DC, USA, pages 368-370, S. TOWNSEND ET AL. 'Tumor rejection after direct costimulation of CD8+ T cells by B7-transfected melanoma cells.' cited in the application see the whole document | 12-16 |
| A | EP,A,0 193 769 (BAYER AG) 10 September 1986 see examples see claims | 28 |

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | WO,A,93 20691 (TYKOCINSKI ET AL.) 28 October 1993<br>see examples<br>see claims | 1-61 |
| A | SCIENCE,<br>vol. 259,no. 5091, 1 January 1993<br>WASHINGTON DC, USA,<br>pages 94-97,<br>J. TROJAN ET AL. 'Treatment and prevention of rat glioblastoma by immunogenic C6 cells expressing antisense insulin-like growth factor I RNA.'<br>see abstract<br>see page 96, right column, line 1 - line 18 | 1-61 |
| P,X | SCIENCE,<br>vol. 263,no. 5146, 28 January 1994<br>WASHINGTON DC, USA,<br>pages 518-520,<br>Y. GUO ET AL. 'Effective tumor vaccine generated by fusion of hepatoma cells with activated B cells.'<br>see the whole document | 1-61 |

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 94/ 14297

**Box I** Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
see annex

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II** Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

Remark: Although claims 1-48 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Although claim 50(completely) and claims 49 and 51(partially, as far as an in vivo method is concerned) are directed to a diagnostic method practised on the human/animal body, the search has been carried out and based on the alleged effects of the compound/composititon.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Interr ial Application No
PCT/US 94/14297

| Patent document cited in search report | Publication date | Patent family member(s) | | Publication date |
|---|---|---|---|---|
| EP-A-203403 | 03-12-86 | JP-A- | 61254527 | 12-11-86 |
| | | JP-A- | 62010016 | 19-01-87 |
| | | JP-A- | 62012718 | 21-01-87 |
| | | JP-A- | 62032879 | 12-02-87 |
| | | DE-A- | 3687014 | 03-12-92 |
| WO-A-9307906 | 29-04-93 | CA-A- | 2121127 | 29-04-93 |
| EP-A-193769 | 10-09-86 | DE-A- | 3507398 | 04-09-86 |
| | | JP-A- | 61205488 | 11-09-86 |
| WO-A-9320691 | 28-10-93 | AU-B- | 4106393 | 18-11-93 |
| | | EP-A- | 0637201 | 08-02-95 |